

PATENT
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SUBSTITUTE SPECIFICATION
for
UNITED STATES LETTERS PATENT APPLICATION
for
DELIVERY OF TREFOIL PEPTIDES
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CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a § 371 national stage filing of PCT/EP00/06343, filed July 5, 2000, which claims priority to EP 99870143.7 filed July 5, 1999.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of *in vivo* protein delivery systems. More particularly, the present invention relates to the secretion *in vivo* of trefoil peptides by microorganisms, preferably bacterial strains, preferably non-pathogenic strains, preferably non-invasive strains, preferably food grade strains, methods for delivering trefoil peptides using said systems and the use of said trefoil peptide expression systems for treatment of inflammatory disorders of the gastro-intestinal tract

DESCRIPTION OF RELATED ART

[0003] *Lactococcus lactis* is a Gram-positive non-pathogenic lactic acid bacterium which can survive in the intestine (Klijn et al., 1995). It is not certain whether *L. lactis* can also be metabolically active in all of these environments.

[0004] The expression of tetanus toxin fragment C by *Lactococcus lactis* in view of vaccination was described by Wells et al. (1993) and Robinson et al. (1997). Further, it was demonstrated that when preparations of *L. lactis* bacteria engineered to express either Interleukin-2 or Interleukin-6 together with tetanus toxin fragment C (TTFC) were administered intranasally to mice, more than 10 times more anti-TTFC was produced than after similar administration of strains expressing TTFC alone (International patent application published under WO 97/14806). These results prove the use of a cytokine-secreting, non-invasive experimental bacterial vaccine vector to enhance immune responses to a co-expressed antigen. Also an approach has been described to attach heterologous protein fragments in the cell wall and by this way display them at the *L. lactis* surface, possibly leading to more enhanced vaccination properties (WO 97 09437 Steidler, Remaut, Wells).

[0005] Trefoil peptides are secreted by epithelial mucus cells and are stable in an acid environment. These peptides contribute to the protection of the mucosa (formation of a gel over the epithelium) and are probably involved in the repair of damaged mucosa by

stimulation of epithelial migration (Playford et al., 1996). The production of trefoil peptides increases locally in regions where damage occurs such as gastric ulcers and colitis (Wright et al., 1990). Babyatsky et al. (1996) have shown that the administration of recombinant trefoil peptides reduces the damage at those places. In contradiction with most other proteins that are important for the protection of the mucosa (such as epidermal growth factor), most studies have demonstrated that trefoil peptides cause little or no proliferation (Playford et al., 1996). Three members of this family of trefoil peptides have been identified in humans and originally designated: pS2 (breast cancer oestrogen inducible gene, O. Lefebvre, 1993), SP (spasmolytic peptide) and ITF (intestinal trefoil factor). In the present nomenclature pS2 is renamed as TFF1, SP as TFF2 and ITF as TFF3 (see e.g. Wong et al., 1999). This new nomenclature will be used throughout the present text.

[0006] In humans, mice and rat TFF1 and TFF2 are predominantly found in the stomach while TFF3 is predominantly found in the duodenum and colon. Wong et al. (1999) give a recent overview of trefoil peptides. The contents of this article are incorporated by reference in the present disclosure. TFF1 is thought to act through a cell surface receptor (Tan et al., 1997).

[0007] The use of trefoil proteins or peptides for treatment of disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal are described in WO 97/38712 and WO 92/14837. These proteins can be used either to treat lesions in these areas or to inhibit the formation of lesions. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, Crohn's disease, ulcerative colitis and acute colitis of chemical, bacterial or obscure origin. Trefoil peptides are particularly useful to treat acute colitis.

[0008] ITF has also been used in combination with EGF (epidermal growth factor) for treating gastro-intestinal tract ulcers. In vitro and in vivo experiments have shown that the

wound healing activities of EGF are markedly increased by treatment of EGF in combination with ITF, without increasing the proliferative action of EGF (Chinery and Playford, 1995).

[0009] Inflammatory bowel disease is the group name for a range of gastro-intestinal inflammations. Belonging to this group are enteritis, colitis, inflammations of respectively the mucosa of the duodenum or the colon. Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa) are closely related, chronic and spontaneously recurring diseases of the gastro-intestinal tract. These diseases are immunologically mediated and have environmental and genetic causes. Sartor (1995) describes the different aspects of inflammatory bowel disease. Crohn's disease has been particularly studied by for instance Herfath and Sartor, (1994), Cominelli et al. (1994), and MacDermott (1989).

[0010] The aim of the present invention is to provide a method for delivering trefoil peptides to treat gastro-intestinal disorders.

[0011] Another aim of the present invention is to provide a pharmaceutical composition for treating gastro-intestinal disorders.

[0012] The present invention relates more particularly to a microorganism delivering a trefoil peptide in vivo. Preferentially said microorganism is a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, more preferably a gram-positive bacterial strain, most preferably a lactic acid fermenting bacterial strain, preferably a *Lactococcus* or a *Lactobacillus* species expressing a trefoil peptide in vivo. The present invention is thus applicable to any of the *Lactococcus* or *Lactobacillus* species or subspecies selected from the list comprising *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis*, *Lactococcus lactis* subsp. *Lactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Lactobacillus acetotolerans*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *Lactobacillus aviarius* subsp. *araffinosus*, *Lactobacillus aviarius* subsp. *aviarius*, *Lactobacillus bavaricus*, *Lactobacillus bif fermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus bulgaricus*, *Lactobacillus carnis*, *Lactobacillus casei*, *Lactobacillus casei* subsp. *alactosus*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei*

subsp. pseudopiantarum, *Lactobacillus casei* subsp. rhamnosus, *Lactobacillus casei* subsp. tolerans, *Lactobacillus cateniformis*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus confusus*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis* subsp. coryniformis, *Lactobacillus coryniformis* subsp. torquens, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus curvatus* subsp. curvatus, *Lactobacillus curvatus* subsp. melibiosus, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii* subsp. bulgaricus, *Lactobacillus delbrueckii* subsp. delbrueckii, *Lactobacillus delbrueckii* subsp. lactis, *Lactobacillus divergens*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus fructosus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus halotolerans*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus iners*, *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kandleri*, *Lactobacillus kefir*, *Lactobacillus kefirianofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus kunkeei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus minor*, *Lactobacillus minutus*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei* subsp. paracasei, *Lactobacillus paracasei* subsp. tolerans, *Lactobacillus parakefiri*, *Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus piscicola*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus rimae*, *Lactobacillus rogosae*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* subsp. carnosus, *Lactobacillus sakei* subsp. sakei, *Lactobacillus salivarius*, *Lactobacillus salivarius* subsp. salicinius, *Lactobacillus salivarius* subsp. salivarius, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus suebicus*, *Lactobacillus trichodes*, *Lactobacillus uli*, *Lactobacillus vaccinofermentans*, *Lactobacillus vaginalis*, *Lactobacillus viridescens*, *Lactobacillus vitulinus*, *Lactobacillus xylophilus*, *Lactobacillus yamanashiensis*, *Lactobacillus yamanashiensis* subsp. mali, *Lactobacillus yamanashiensis* subsp. Yamanashiensis and *Lactobacillus zeae*.

[0013] It was not obvious from the capacity of *Lactococcus lactis* to deliver one heterologous antigen or its ability to produce molecules such as IL-2 and IL-6 in vitro and in vivo that bacteria would be an appropriate vehicle for delivery of other types of peptides or

polypeptides *in vivo*. Further it is unknown whether said trefoil peptides expressed by said bacterial strains will provide a beneficial effect to inflammatory diseases of the gastro-intestinal tract, such as inflammatory bowel disease or acute colitis.

[0014] It is, therefore, surprising that it could be demonstrated in the present Examples section that bacterial strains are able to express trefoil peptides *in vivo* when present in the gastro-intestinal canal and exert a healing effect in acute colitis situations. By way of example, PCR fragments containing the coding region mouse TFF1 were cloned. Recombinant vectors comprising these PCR clones under the control of a promotor and the *usp45* *Lactococcus lactis* secretion signal sequence were constructed. Transformed *Lactococcus lactis* strains were constructed which express mouse TFF1 trefoil peptides. It was further shown in an *in vivo* mice model system that recombinant mTFF1 produced by these bacteria can surprisingly exert healing effects on the distal part of the inflamed colon.

SUMMARY OF THE INVENTION

[0015] According to a preferred embodiment, the present invention relates particularly to a bacterial strain delivering trefoil peptide *in vivo*.

[0016] According to another preferred embodiment, the present invention relates to a bacterium delivering TFF1 *in vivo*.

[0017] It is to be understood that the present invention also relates to parts or variants of any trefoil peptide. Said parts refer to biologically active parts which can be generated by methods known to those skilled in the art. These parts will generally contain at least 10 contiguous amino acids, typically at least 20 contiguous amino acids, more typically at least 30 contiguous amino acids, usually at least 40 contiguous amino acids, and preferably at least 50 contiguous amino acids. Said variants refer to variants which have the same biological activity as the above mentioned trefoil peptides.

[0018] It should also be clear that bacterial strains according to the present invention as defined above, may also express additional recombinant proteins which are beneficial to the treatment of any envisaged disorder.

[0019] According to yet another embodiment, the present invention relates to a pharmaceutical composition comprising a microorganism expressing a trefoil peptide as defined above.

[0020] Advantageously, the pharmaceutical composition according to the present invention is preferably suitable for application to mucosal surfaces.

[0021] Pharmaceutical compositions according to the present invention, and for use in accordance to the present invention, may comprise, in addition to the microorganism, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration. Those of relevant skill in the art are well able to prepare suitable solutions.

[0022] According to another embodiment, the present invention relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a microorganism as defined above.

[0023] According to another aspect, the present invention also relates to the use of a microorganism as defined above for the manufacture of an agent for the delivery of trefoil peptide to the gastro-intestinal tract.

[0024] According to another embodiment, the present invention relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a microorganism as defined above.

[0025] The present invention also relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a microorganism delivering a TFF1 trefoil peptide *in vivo*.

[0026] The trefoil proteins expressed by the bacterial strains according to the present invention can be used either to treat lesions in these areas or to inhibit the formation of lesions caused by gastro-intestinal diseases and disorders.

DESCRIPTION OF THE FIGURES

[0027] The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these figures in combination with the detailed description of specific embodiments presented herein.

[0028] Figure 1A is a schematic map of plasmids pL2mTFF1v1. T7 is the major late promoter from coliphage T7 (Studier and Moffatt, 1986). P1 is the lactococcal promoter as in Waterfield et al., (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 protein (van Asseldonck et al., 1990), mtff1 is a DNA fragment encoding the mature part of murine TFF1, mtff1v1 is a DNA fragment encoding a truncated (missing two aminoterminal aa residues) mature murine TFF1, Cm is the chloramphenicol selection marker, Em is the erythromycin selection marker.

[0029] Figure 1B is a schematic map of plasmid pT1mTFF1.

[0030] Figure 1C is a schematic map of plasmid pPICmTFF1. PPMF is the prepro *Saccharomyces cerevisiae* α -mating factor; AOX1 prom is the alcohol oxidase promoter; AOX1 term is the alcohol oxidase terminator; HIS4 is the Histidol dehydrogenase gene; Ori is an Escherichia coli origin of replication; AOXfr is a 3' fragment of the alcohol oxidase gene; AmpR is the ampicillin resistance gene. All components are from the pPIC9 plasmid (Invitrogen).

[0031] Figures 2A-2B are the DNA sequence of plasmid pL2mTFF1v1 (SEQ ID NO 1).

[0032] Figures 3A-3C are the DNA sequence of plasmid pT1mTFF1 (SEQ ID NO 2).

[0033] Figures 4A-4C are DNA sequence of plasmid pPICmTFF1 (SEQ ID NO 3).

[0034] Figure 5 is an SDS-PAGE. The different protein fractions are derived from the medium of *L. lactis* MG1820 [pILPOL] (control), MG1820 [pILPOL; pL2mTFF1v1] , MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells. The two left lanes contain marker proteins wherein the molecular weight is given in kDa. The proteins were visualised using Coomassie Blue® staining.

[0035] Figure 6A is a representation of the histological scores of the distal part of the colon, showing epithelium damage (distal part colon).

[0036] Figure 6B is a representation of the histological scores of the distal part of the colon, showing inflammatory infiltration (distal part colon).

[0037] Figure 6C is a representation of the histological scores of the distal part of the colon, showing the sum of the histological scores of the top graphics (distal part colon).

[0038] Figure 7 is a representation of the histological scores of the distal part of the colon of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment with MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.

[0039] Figure 8A shows pro-inflammatory cytokine titrations in acute inflamed colon tissue, specifically interleukin-1 β in distal colon of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment with MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.

[0040] Figure 8B shows pro-inflammatory cytokine titrations in acute inflamed colon tissue, specifically interferon- γ in middle and distal colon (right) of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment with MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.

[0041] Figure 9 is an SDS-PAGE of protein fractions from the medium of selected *Pichia pastoris* (GST115::pPICmTFF1) and negative control. The mTFF1 producer clone which was

further used for production of mTFF1 is indicated by an arrowhead The proteins were visualised using Coomassie Blue ® staining.

[0042] Figure 10A is a Gelfiltration pattern of purified mTFF1 (SUPERDEX 75®; Pharmacia). The mTFF1 protein eluted in two peaks with the majority being present in fractions 14, 15, 16 (dimer) and 20 (monomer). The identity of the protein in these fractions was shown to be mTFF1 by SDS-PAGE (insert). The proteins were visualised using Coomassie® Blue staining.

[0043] Figure 10B illustrates reducing and non reducing SDS-PAGE of purified mTFF1. Left lanes are size markers of indicated sizes, Coomassie® brilliant blue staining.

[0044] Figure 11 is a representation of the histological scores of the distal part of the colon of mice treated by intraperitoneal injection (i.p.), oral (oral) and rectal (rectal) inoculation, before (pre), during (du) or after (po) installation of acute DSS-induced colitis. DSSdu represents scores of PBS treated mice induced for acute DSS colitis.

DEFINITIONS

[0045] The expression “gastric and/or intestinal diseases and/or disorders” relates to all types of gastric, intestinal and gastro-intestinal diseases and/or disorders. In preferred embodiments of the invention this expression relates to acute gastro-intestinal inflammatory diseases and disorders. These diseases are preferably acute gastro-intestinal disorders of chemical, bacterial or obscure origin. Belonging to this group are enteritis, colitis, including but not limited to acute flare-ups in Crohn’s disease and ulcerative colitis inflammations of, respectively, the mucosa of the duodenum or the colon. Also included herewith is traveller’s disease. In other preferred embodiments of the invention the expression “gastric and/or intestinal diseases and/or disorders” relates to chronic and spontaneously recurring diseases of the gastro-intestinal tract such as Crohn’s disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

[0046] The expression “gastric and/or intestinal diseases and/or disorders” also relates to diseases involving lesions at mucosal surfaces. As such, the disease states to be treated by the methods and pharmaceutical compositions of the invention can also include disorders of and

damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, and Crohn's disease.

DETAILED DESCRIPTION OF THE INVENTION

[0047] The present invention thus relates to the use of a microorganism as described above for the preparation of a medicament for treatment of gastric and/or intestinal diseases and/or disorders.

[0048] The present invention also relates to the use of a microorganism as described above for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases, acute colitis, acute flare-ups of Crohn's diseases and ulcerative colitis, and for treatment of chronic and spontaneously recurring diseases of the gastro-intestinal tract comprising Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

[0049] According to another embodiment, the invention relates to the use of a microorganism as described above for the preparation of a medicament for inhibiting the formation of lesions caused by gastric and/or intestinal diseases and disorders.

[0050] Administration of the microorganism may be orally or by means of any other method known in the art allowing the microorganism to enter the desired places to be treated, such as e.g. anal, vaginal. The microorganism may be applied in a nutrient medium, i.e. a medium containing a substance or substances which sustain (at least in vitro) metabolic activity of the microorganism. Such substances may sustain viability if not growth of the microorganism. Such substances may include an energy source such as glucose, amino acids and so on.

[0051] The individual to which the microorganism is administered may be a human or an animal.

[0052] In a therapeutic context, i.e. where the biological effect of delivery of the polypeptide to an individual is beneficial to that individual, administration is preferably in a 'therapeutically effective amount', this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of one symptom. The actual amount administered, and rate and time-course of administration, will depend on the aim of the administration, e.g. the biological effect sought in view of the nature and severity of the challenge and is the subject of routine optimisation. Prescriptions of treatment, for example decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

[0053] A composition comprising microorganisms according to the present invention may be administered in accordance with the present invention alone or in combination with other treatments, either simultaneously or sequentially.

[0054] According to another embodiment, the present invention relates to a method for producing a microorganism delivering a trefoil peptide in vivo as defined above comprising transforming a microorganism with a recombinant vector carrying a trefoil polypeptide coding sequence under the control of a suitable promoter and a suitable bacterial secretion signal sequence.

[0055] Said bacterial secretion signal sequence can be any sequence known in the art to perform said function. Preferably, for *L. lactis* said secretion signal is the *usp45* *L. lactis* secretion signal sequence. Said promoter sequence can be any promoter allowing expression of said coding sequence in said microorganism. Examples given in the examples section include the known inducible *E. coli* phage T7 promoter and the known constitutive P1 promoter of *L. lactis*.

[0056] The present invention also relates to a recombinant vector comprising at least a part of a trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence. Said recombinant vector can be used to deliver in vivo at least a part of a trefoil peptide sequence which can exert on healing effect on damaged areas of the mucosal surfaces.

[0057] The present invention further relates to a recombinant vector as defined above, having a nucleotide sequence as represented by any of SEQ ID NOs 1, 2 or 4.

[0058] The following examples merely serve to illustrate the present invention, and are not to be construed as limiting the invention in any way. All documents mentioned in this text are incorporated by reference.

EXAMPLES

Example 1: Cloning and expression of mouse TTF1 (mTTF1)

Culture media

[0059] GM17 is M17 (Difco, Detroit) supplemented with 0.5 w/v % of glucose. M9 medium contains per litre: 6g of Na₂HPO₄, 3 g of KH₂PO₄, 1 g of NH₄Cl, 0.5 g of NaCl, 2 mmol of MgSO₄, 0.1 mmol of CaCl₂ and 5 g of Casitone (Difco). M9B is M9 supplemented with 2.1 g of NaHCO₃ and 2.65 g of Na₂CO₃ per liter. GM9B is M9B supplemented with 0.5 w/v % of glucose. LM9B is M9B supplemented with 0.5 w/v % of lactose.

[0060] When appropriate the antibiotics, erythromycin (Er) or chloramphenicol (Cm), were added to the respective media at final concentrations of 5 µg/ml each. The designation used to indicate the presence of antibiotic is, e.g. GM17Er, LM9BCm and so on. Solid media contained 1.2 % agar.

Recombinant DNA techniques

[0061] DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out according to standard procedures. *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells et al., 1993a). Plasmid DNA was routinely purified using the Qiagen Plasmid Kit

PCR amplification of mTFF1

[0062] The PCR reaction was carried out on a plasmid containing mTFF1 cDNA (Lefebvre, 1993) using the oligonucleotide primers mTFF1S and mTFF1A. The mTFF1S primer corresponds to the first 18 nucleotides of the sense strand of mTFF1 from the first nucleotide behind the signal sequence. The mTFF1A primer is complementary to the last 26 nucleotides of the sense strand of mTFF1 including the stop codon, and introduces an extra SpeI restriction site.

mTFF1S: 5'-CAGGCCCAGCCCAGGCC -3' (SEQ ID NO 4)

mTFF1A: 5'-GCACTAGTTAGAAGGGACATTCTTCTTCTTG AG-3' (SEQ ID NO 5) wherein ACTAGT in mTFF1A represents an SpeI site.

[0063] PCR amplification was carried out using VENTTM DNA polymerase (New England Biolabs, Beverly, USA) which gives a PCR product carrying blunt ends. The PCR mixture consisted of 2 units Vent DNA polymerase, 10µl VENT buffer (thermopol), 4µl dXTP's (0.5mM maximum), 5µl (0.5µM) of each primer, 1µl (50 ng) template DNA and 74µl H₂O. Six reactions were set up differing in their final concentration of MgSO₄, adjusted to 0, 1, 2, 3, 4 and 5 mM respectively. PCR amplification cycles were: T₀ for 300'' at 94°C, T₁ for 45'' at 94°C, T₂ for 30'' at 60°C, T₃ for 20'' at 72°C, T₄ for 10'' at 20°C. These cycles T₁ until T₃ were carried out 30 times.

[0064] PCR amplification with these primers rendered the gene for mature mTFF1 lacking the signal sequence and including an additional SpeI restriction site. After checking by gel electrophoresis, the amplified fragment appeared as a band in the expected length range. The 5' end of the mTFF1 sequence contains two possible target sequences complementary to the forward primer. As a consequence two fragments of 202 base pairs and 208 base pairs respectively can be amplified from the mTFF1 cDNA by use of the mentioned primers. These fragments are not expected to be resolved by agarose gel electrophoresis.

Construction of plasmids

[0065] Two different types of vectors were used as acceptors for the mTFF1 trefoil peptide encoding PCR fragment. The primary structure of the two parental vectors - pT1NX, derived from pTREX1 (Wells and Schofield, 1996), and pLET2NX, derived from pLET2N (Steidler

et al., 1995) - contains the following common elements: a promoter (T7 or P1), the *L.lactis* usp45 secretion signal sequence (van Asseldonk et al., 1990 and European patent application published under No. 0 455 280), modified to contain a *NaeI* restriction site overlapping the sequence encoding the ultimate aa residue (Steidler et al., 1995), and a downstream *SpeI* restriction site. pT1NX derived plasmids specify resistance to erythromycin; pLET2NX derived plasmids specify resistance to chloramphenicol. The PCR fragments were treated for 1 hour at 37°C using 50µl DNA solution, 10µl *SpeI*-buffer, 50 units *SpeI*, 10 units T4 polynucleotide kinase (Gibco BRL, Bethesda, USA), 0.5 mM ATP, adjusted to pH 7.5, and 36µl H₂O. The vector pT1NX was digested for 1 hour at 37°C using 10 à 20µl purified DNA, 10µl *NaeI* buffer, 10 units *NaeI*, 50 units *SpeI*, 1 unit calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany) and 73 à 63µl H₂O. After 30 minutes incubation, 50 units of *SpeI* and 10 units of *NaeI* were again added to the mixture. The restriction enzymes were inactivated and extracted from the mixture by phenol/chloroform extraction. After restriction digestion, the mTFF1-derived band (comprising a 195 bp and a 201 bp fragment as described before under "PCR amplification of mouse TFF1 (mTFF1)", and the vector parts were excised from the agarose gel. Following ligation of the respective PCR fragments and the vector for 45 minutes at 16°C using "Ready To Go" T4 DNA ligase (Pharmacia Biotech, UK) recombinant plasmids were obtained containing the mTFF1 cistron as an in-frame fusion to the usp45 secretion signal sequence under the control of the promoter.

[0066] The plasmid pT1mTFF1 (Figure 1B), which contains the constitutive *L. lactis* P1 promoter, resulted from ligation of the purified *NaeI* - *SpeI* vector part of pT1NX and the *SpeI* cut and 5' phosphorylated PCR fragment.

[0067] The plasmid pL2mTFF1v1 (Figure1A), which contains the inducible *E. coli* phage T7 promoter, resulted from ligation of the purified *NaeI* - *SpeI* vector part of pLET2N and the *SpeI* cut and 5' phosphorylated PCR fragment. The T7 promoter can only be activated by the cognate T7 RNA polymerase encoded by e.g. plasmid pILPOL. This plasmid is present in *L. lactis* strain MG1820 [pILPOL] (Wells et al., 1993c).

[0068] For structural analysis plasmid pT1mTFF1 was transformed into *L. lactis* strain MG1363. The cells were grown on GM17Er plates. Colonies were grown in 2.5 ml GM17Er and the plasmid was isolated. By means of an analytical digest, the restriction pattern of the pT1NX vector (2µl DNA (pT1NX), 20 units *EcoRI*, 50 units *SpeI*, 2µl *SpeI*-buffer and 15µl

H₂O) and the isolated recombinant plasmid (5µl DNA, 20 units EcoRI, 50 units SpeI, 2µl SpeI-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H₂O) were compared. The plasmids were cut with EcoRI and SpeI for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4999bp are predicted. In pT1mTFF1, two bands of 499 bp and 4999 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From each recombinant plasmid, one positive culture was streaked out on GM17Er plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Er medium and grown to saturation. The cells were collected and the plasmids were purified. Their physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the mTFF1 cistron had been ligated perfectly in frame with the usp45 secretion leader sequence. pT1mTFF1 contains a 208 bp insert which represents the complete coding sequence of mature mTFF1 (as described before under “PCR amplification of mouse TFF1 (mTFF1)”).

[0069] For structural analysis plasmids pL2mTFF1v1 was transformed into strain MG1820[pILPOL]. The cells were grown on GM17Cm plates. Colonies were grown in 2.5 ml GM17Cm and the plasmids were isolated. By means of an analytical digest, the restriction pattern of the pLET2NX vector (2µl DNA (pLET2NX), 20 units EcoRI, 50 units SpeI, 2µl SpeI-buffer and 15µl H₂O) and the isolated recombinant plasmid (5µl DNA, 20 units EcoRI, 50 units SpeI, 2µl SpeI-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H₂O) were compared. The recombinant plasmid was cut with EcoRI and SpeI for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4650bp are predicted. In pL2mTFF1, two bands of 499 bp and 4650 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From the recombinant plasmid, one positive culture was streaked out on GM17Cm plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Cm medium and grown to saturation. The cells were collected and the plasmid was purified. Its physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the mTFF1 cistron had been ligated in frame with the usp45 secretion leader sequence. The analysis further showed that pL2mTFF1v1 contains a 202 bp insert (consequently missing the first two aminoterminal aa residues of mature mTFF1; as described before under “PCR amplification of mouse TFF1 (mTFF1)”). The sequences of the recombinant plasmids are given in figures

2A-2B and 3A-3C. Their complete sequences were compiled from the published sequences of the constituting parts. In addition, relevant sections of the sequences such as PCR fragments and ligation junction points were experimentally verified.

Protein expression in transformed L. lactis

[0070] *L. lactis* strains were transformed with the plasmids as constructed above. For transformation of the pT1mTFF1 plasmid, *L. lactis* strain MG1363 (Gasson, 1983) was used. For transformation of the pL2mTFF1v1 plasmid, *L. lactis* strain MG1820 (pILPOL) (Maeda and Gasson, 1986) was used.

[0071] The expression of the proteins by these transformed *L. lactis* strains was detected by SDS-PAGE.

[0072] To prepare culture supernatant fractions, the cells were grown for 20 hours at 28°C in five ml GM17Er medium for the pT1mTFF1 plasmid or GM17Cm medium for the pL2mTFF1v1 plasmid. The cultures were diluted 1/100 in five ml of either GM17Er or GM17Cm medium and grown for 3 hours at 28°C. The cells were collected by centrifugation at 2800 rpm for 20 min and resuspended in five ml of the appropriate medium, i.e., GM9BEr for MG1363 cells or LM9BCm for MG1820 [pILPOL] cells. After a further five hours of growth the cells were pelleted. The proteins present in the medium fractions were recovered by phenol extraction and ethanol precipitation.

[0073] The proteins expressed in the culture supernatant fraction of a *L. lactis* MG1820 control strain compared to *L. lactis* MG1820 strains transformed with [pILPOL; pL2mTFF1v1] and *L. lactis* MG1363 transformed with [pTREX1; pT1mTFF1] are shown in Figure 5. This figure shows an extra protein band of the appropriate size (indicated by the arrowhead) in MG1820 [pL2mTFF1v1] and MG1363[pT1mTFF1] when compared with the controls. As can be observed from this figure, the expression of the recombinant gene is quite low. This renders the observed in vivo result surprising since others use purified trefoil peptides in therapies for the repair of gastric and intestinal injury at dramatically higher levels; e.g. Tran et al. (1999) used daily intrarectal application of human recombinant TTF2 at levels of 2.5 mg/kg body weight for five days to obtain a reduction in the inflammatory index of experimentally installed colitis in rats (intracolonic administration of dinitrobenzene sulphonic acid in alcohol).

Example 2: *In vivo* testing of MG1363 [pT1mTFF1]

Preparation of cells for intragastric administration

[0074] Transformants of *L. lactis* strains, MG1363 [pTREX1], MG1363 [pT1mTFF1] were streaked on GM17Er plates and grown overnight at 28°C. In each case a single colony was subsequently grown overnight at 28°C in 15 ml GM17Er medium. To this culture, 15 ml 100% glycerol was added in order to preserve said cells at -20°C. Each day, the necessary amount of cells could be inoculated for treatment of mice. To this end the culture was diluted 1/200 into 10 ml GM17Er medium. After minimum 20 hours of growth at 30°C, the cells were collected by centrifugation for 15 min at 2800 rpm. The cells were then resuspended in 1 ml M9B without antibiotic.

In vivo tests in mice with acute colitis

[0075] The effect of the trefoil peptides expressed from these *L. lactis* bacteria was tested out in mice suffering from acute colitis. Twenty-one female Balb/c mice received 5% DSS (dextrane sodium sulphate) dissolved in their drinking water during 7 days. In this manner, acute colitis was induced (Kojouharoff et al., 1997). For therapeutic purposes these mice were orally inoculated daily by means of a gastric catheter using 100µl bacterial suspension (minimum 1.10^8 cells) from day 1 until day 7 of the DSS treatment. As indicated Six mice were inoculated with MG1363 [pTREX1] cells, six mice were inoculated with MG1363 [pT1mTFF1] cells and three mice were not inoculated (DSS control). On day 8 after the induction of colitis, the mice were sacrificed and examined immunologically and histologically.

[0076] Immunological testing of the sera showed that the treated mice did not show an immune response towards the expressed proteins. Serum was taken from the mice which were bled at day 8. This serum was analysed *via* Western blotting to check whether it contained antibodies against the proteins present in the medium fractions of the *L. lactis* cells. The medium fractions used were derived from the *L. lactis* strains MG1363 [pTREX1] and MG1363 [pT1mTFF1]. An equivalent of 1 ml of concentrated (phenol extraction and ethanol precipitation) medium fractions were analysed by SDS-polyacrylamide (20%) gel electrophoresis. After blotting to nitrocellulose filters, the filters were incubated for 1 hour with the serum solutions of the 4 groups of mice. The serum was diluted 500 times in 20ml

nitrocellulose blocking buffer (Blotto: 100ml 10x PBS, 150ml 1M NaCl, 2ml TRITON X-100®, 25g fat-free milkpowder, water up to a total volume of 1 liter). As a secondary antibody, sheep anti-mouse IgG coupled to horseradish peroxidase (HRP) was used. Using the 500 times diluted serum, no signal was detected.

[0077] Histological analysis was performed on colons of the treated mice. The colons were cut in the length direction and divided in three equal portions: the distal (nearest to the anus), middle and proximal parts. These colon parts were analysed histologically after an overnight fixation in 3.7% formaldehyde (in PBS), followed by paraffin embedding, ensuring upright positioning of the tissue samples in the paraffin blocks. Of each tissue sample, three parallel 3µm thick longitudinal sections, evenly spaced over the sample, were made. These crossections were coloured with hematoxylin/eosin. Histological analysis was performed in a blind fashion, meaning that the labels on the slides were covered before scoring the sections. Slides carrying sections obtained from the several groups of mice were randomized before microscopic examination. Each slide was then assigned a histological score (ranging from 0 to 5) according to the symptomatic description as defined in Table 1.

Table 1. Symptomatic description of histological scores.

Score	Epithelium damage	Inflammatory infiltration*
0	Normal morphology	No infiltration
1	Loss of a few goblet cells	Infiltration around the basis of the crypts
2	Widespread loss of goblet cells	Infiltration which reaches the Lamina muscularis mucosae
3	Loss of crypts	Extensive infiltration which reaches the Lamina muscularis mucosae and thickening of the mucosa with prominent oedema
4	Widespread loss of crypts	Infiltration which reaches the Lamina submucosa

* Inflammatory infiltration includes infiltration of the granulocytes, macrophages and lymphocytes.

[0078] For each mouse and for each colon part, the average score of the three sections was calculated. In the distal and middle parts of the colon, the inflammation consisting of epithelial damage and infiltration were the most pronounced. In the proximal part, almost no inflammation could be observed. The average histological score was calculated for both the distal and the middle colon part per group of animals. The final histological sum score is the

sum of the two separate scores (sum score = score of epithelial damage + score of infiltration) and is a measure for the degree of the inflammation. The histological sum scores of the distal colon part for each of the groups of mice is shown in Figures 6A-6C.

[0079] From the histological scores for the distal part of the colon as set out in Figures 6A-6C, it could be concluded that there is a clear decrease of inflammation upon inoculation of mice with *L. lactis* cells producing trefoil peptides. Mice having received [pT1mTFF1] transformed *L. lactis* cells show a significant reduction of the inflammation of more than 65%.

[0080] As can be seen from Figures 6A-6C, the inflammatory infiltration and the epithelial damage in the distal part of the colon are significantly decreased following inoculation with recombinant *L. lactis* strains which secrete mTFF1 polypeptide

[0081] These results were confirmed in a separate experiment which was conducted equally, including larger groups (group size = 10) and more control groups. Figure 7 shows histological scores (obtained as described above) of healthy control mice (control) and of mice which received DSS as described, either left untreated (DSS) or treated (as described above) with MG1363, MG1363 [pT1TREX1] or MG1363 [pT1mTFF1] as indicated. The experiment shows a clear and significant decrease in the intestinal inflammation in the group of mice treated with MG1363 [pT1mTFF1]

[0082] The latter experiment was also evaluated by determining the levels of interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ), both pro-inflammatory cytokines well known to the skilled. Mice (n=10) were inoculated with the strains indicated as described. Control = healthy mice, DSS = mice receiving 5% DSS in the drinking water without any treatment. The colon was prepared out and areas with equal surface were isolated by means of a punch (\varnothing = 4 mm). The tissue samples of each group were overlayed with 500 μ l RPMI + 10% fetal calf serum and incubated overnight at 37°C. The supernatant was collected and titrated for cytokine content by ELISA. The amount of IL-1 β and IFN- γ in the respective tissues is shown in Figures 8A-8B. The results show a clear reduction in these pro-inflammatory cytokines in groups of mice treated with MG1363 [pT1mTFF1].

Example 3: Comparison of treatment with MG1363 [pT1TFF1] and purified TFF1

Construction of plasmids

[0083] For the expression of mTFF1 from *Pichia pastoris* we constructed the plasmid pPICmTFF1 (Figure 1C). For this, the mTFF1 gene was PCR amplified as described (PCR amplification of mouse TFF1). This fragment was ligated in the opened *NaeI* restriction site of a derivative of pPIC9 (Invitrogen). The ligation mixture is transformed to *E. coli* MC1061 and correctly assembled clones were identified by restriction analysis and DNA sequencing (sequence as in Figure 4A-4C). In the resulting plasmid pPICmTFF1, the mTFF1 sequence is fused in frame with the *Saccharomyces cerevisiae* α -mating factor prepro secretion signal

Expression and Purification of mTFF1

[0084] The plasmid pPICmTFF1 was transferred to *Pichia pastoris* GST115 by a method as described in Logghe (1995) and positive clones, which had the mTFF1 unit integrated in the *his4* locus, were selected by PCR identification. These positive clones were induced with methanol and screened for expression by protein analysis of culture supernatant and one clone which showed, when compared to the negative control (negative), a particularly high expression of an extra band at 6,5 kDa (GST115::pPICmTFF1) was retained for further work (Figure 9, indicated by arrowhead). The extra protein band was identified as mTFF1 by protein sequencing. The expression procedure was optimised scaled up and optimised to a 16 l culture and mTFF1 was purified from the culture supernatant.

[0085] For this, methanol induced GST115::pPICmTFF1 supernatants were concentrated by tangential filtration (Millipore proflux M12, cut off 3000 Da) and were dialysed to pH 7.4 in a 0.02 M phosphate buffer. mTFF1 was purified from this concentrate on an ion-exchange column (Q-column of Biorad). The proteins were eluted from the column by an isocratic salt gradient. The resultant mTFF1 was more than 99% pure and was further concentrated. The final preparation contains less than 160 ng LPS /ml. This amount of LPS is within acceptable limits and the pS2 protein can be used in future experiments.

[0086] Following analysis on a size exclusion column of purified mTFF1 (SUPERDEX 75®; Pharmacia) we conclude that 7.5 % of the mTFF1 is in the monomeric form, and 92.5 % is in the dimeric form (Figure 10A). This was confirmed by reducing versus non reducing SDS-PAGE of the purified mTFF1 (Figure 10B).

Assessment of biological activity of purified TFF1

[0087] A well known feature of TFF1 protein is that after administration of the protein to Caco-2 cell monolayers it significantly lowers the surface expression of E-cadherine (Liu et al., 1997). We showed a lowering of 10 % of the E-cadherine surface expression after the above described preparation of mTFF1 was administered to Caco-2 monolayers.

Treatment of murine acute colitis with purified mTFF1:

[0088] For induction of acute colitis mice received 6% dextran sulfate sodium (DSS, MW 40 000) dissolved in drinking water for 7 days (Kojouharoff et al., 1997). Mice used for experiments were age-matched and had received DSS treatment simultaneously. For therapeutic purposes, mice were treated daily with 50 µg mTFF1 in 200 µl PBS before DSS administration from day -7 to 0 (pre-treatment groups), during DSS administration from day 0 to 7 (during-treatment groups) and after DSS administration from day 7 to 14 (post-treatment groups). To study different routes to deliver mTFF1, mice were treated by intraperitoneal (i.p.) injection, intragastric inoculation and rectal administration in each setup. Mice were killed on day 8 after receiving drinking water without DSS for one day (pre-treatment and during-treatment groups) and on day 14 after receiving drinking water without DSS for seven days (post-treatment groups). Non-treated control groups with DSS in drinking water were killed on day 8 and day 14. All groups consisted of 9 mice. Results are represented in Figure 11 and clearly show that in no treatment regime any statistically significant improvement can be observed. This renders the described invention surprising since a clear improvement has been observed (Figures 6A-6C and 7). This means that the delivery of TFF1 through *L. lactis* makes an essential contribution to the observed therapeutic effect.

REFERENCES

- Babyatsky M. W., de Beaumont M., Thim L., Podolsky D. K. (1996). Oral trefoil peptides protect against ethanol- and indomethacin-induced gastric injury in rats. *Gastroenterology* 110, 489-497.
- Chinery R. and Playford R.J. (1995). Combined intestinal trefoil factor and epidermal growth factor is prophylactic against indomethacin-induced gastric damage in the rat. *Clinical Science* 88, 401-403.
- Cominelli F., Kam L., Casini-Raggi V. et al. (1994). Specific mucosal imbalance of IL-1 and IL-1 receptor antagonist (IL-1ra) in IBD: A potential mechanism of chronic inflammation. *Gastroenterology* 106, A667.
- Gasson M.J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* 154, 1-9.
- Herfarth H.H. and Sartor R.B. (1994). Cytokine regulation of experimental intestinal inflammation. *Current Opinion in Gastroenterology* 10, 625-632.
- Klijn N., Weerkamp, A.H., and de Vos W.M. (1995). Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Appl. Environ. Microbiol.* 61 (7), 2771-2774.
- Kojouharoff G., Hans W., Obermeier F., Männel D.N., Andus T., Schölmerich J., Gross V. and Falk W. (1997). Neutralisation of tumor necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin. Exp. Immunol.* 107, 353-358.
- Lefebvre, O., Wolf, C., Kédinger, M., Chenard M.-P., Tomasetto, C., Chambon, P. and Rio, M.-C. (1993). The mouse one P-domain (pS2) and two P-domain (mSP) genes exhibit distinct patterns of expression. *J. Cell. Biol.* 122, 191-198.
- Liu, D., I. el-Hariry, Karayiannakis AJ, Wilding J, Chinery R, Kmiot W, McCrea PD, Gullick WJ, Pignatelli M. (1997). "Phosphorylation of beta-catenin and epidermal growth factor receptor by intestinal trefoil factor." *Lab Invest* 77(6): 557-63.
- MacDermott R.P. (1989). Alterations in serum immunoglobulin G subclasses in patients with ulcerative colitis and Crohn's disease. *Gastroenterology* 96, 764-768.

- Maeda S. and Gasson J.M. (1986). Cloning, expression and location of the *Streptococcus lactis* gene for phospho-B-D-galactosidase. *J. Gen. Microbiol.* 132, 331-340.
- Playford RJ, Marchbank T, Goodlad RA, Chinery RA, Poulson R, Hanby AM (1996). Transgenic mice that overexpress the human trefoil peptide pS2 have an increased resistance to intestinal damage. *Proc Natl Acad Sci U S A.* 93, 2137-2142.
- Robinson K., Chamberlain L. M., Schofield K. M., Wells J. M., Le Page R.W. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature Biotechnol.* 15, 653-657.
- Sartor R.B. (1995). Inflammatory Bowel Disease: Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterology Clinics of North America* Vol. 24, 475-507. W.B. Saunders Company, Philadelphia.
- Sartor R.B. (1995). Inflammatory Bowel Disease: Microbial factors in the pathogenesis of Crohn's disease, ulcerative colitis and experimental intestinal inflammation. *Gastroenterology Clinics of North America.* Vol. 24, 96-124. W.B. Saunders Company, Philadelphia.
- Steidler L., Wells J.M., Raeymaekers A., Vandekerckhove J., Fiers W. and Remaut E. (1995). Secretion of biologically active murine interleukin-2 by *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* 61, 1627-1629.
- Studier F.W. and Moffatt B. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113-130.
- Tan X.-D, Hsueh W., Chang H., Wei, K.R. and Gonzalez-Crussi F. (1997) Characterization of a putative receptor for intestinal trefoil factor in rat small intestine: Identification by in situ binding and ligand blotting. *Biochem. Biophys. Res. Communications* 237, 673-677.
- Tran C.P., Cook G.A., Yeomans N.D., Thim L. and Giraud A.S. (1999). Trefoil peptide TFF2 (spasmolytic polypeptide) potentially accelerates healing and reduces inflammation in a rat model of colitis. *Gut* 44, 636-642.
- van Asseldonck M., Rutten G., Oteman M., Siezen R.J., de Vos W.M. and Simons G. (1990). Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* 95, 155-160.
- Waterfield N.R., Le Page R.W.F., and Wells J.M. (1995). The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis*. *Gene* 165, 9-15.

Wells J.M., Wilson P.W. and Le Page R.W.F. (1993a). Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J. Appl. Bacteriol.* 74, 629-636.

Wells J.M., Wilson P.W., Norton P.M., Gasson M.J. and Le Page R.W.F. (1993b). *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol. Microbiol.* 8, 1155-1162.

Wells J.M., Wilson P.W., Norton P.M., and Le Page R.W.F. (1993c). A model system for investigation of heterologous protein secretion pathways in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 59, 3954-3959.

Wells J.M. and Schofield K.M. (1996). Cloning and expression vectors for lactococci. NATO ASI Series, Vol. H 98, 37-62. *Lactic Acid Bacteria : Current Advances in Metabolism, Genetics and Applications*. T.F. Bozoglu & B. Ray (Eds). Springer-Verlag, Berlin, Heidelberg.

Wong, W.M. (1999). Trefoil peptides. *Gut* 44: 890-895.

Wright N.A., Poulsom R., Stamp G.W., Hall P.A., Jeffery R.E., Longcroft J., Rio M.C., Tomasetto C and Chambon P. (1990). Epidermal growth factor (EGF/URO) induces expression of regulatory peptides in damaged human gastrointestinal tissues. *J. Pathol.* 162, 279-284.